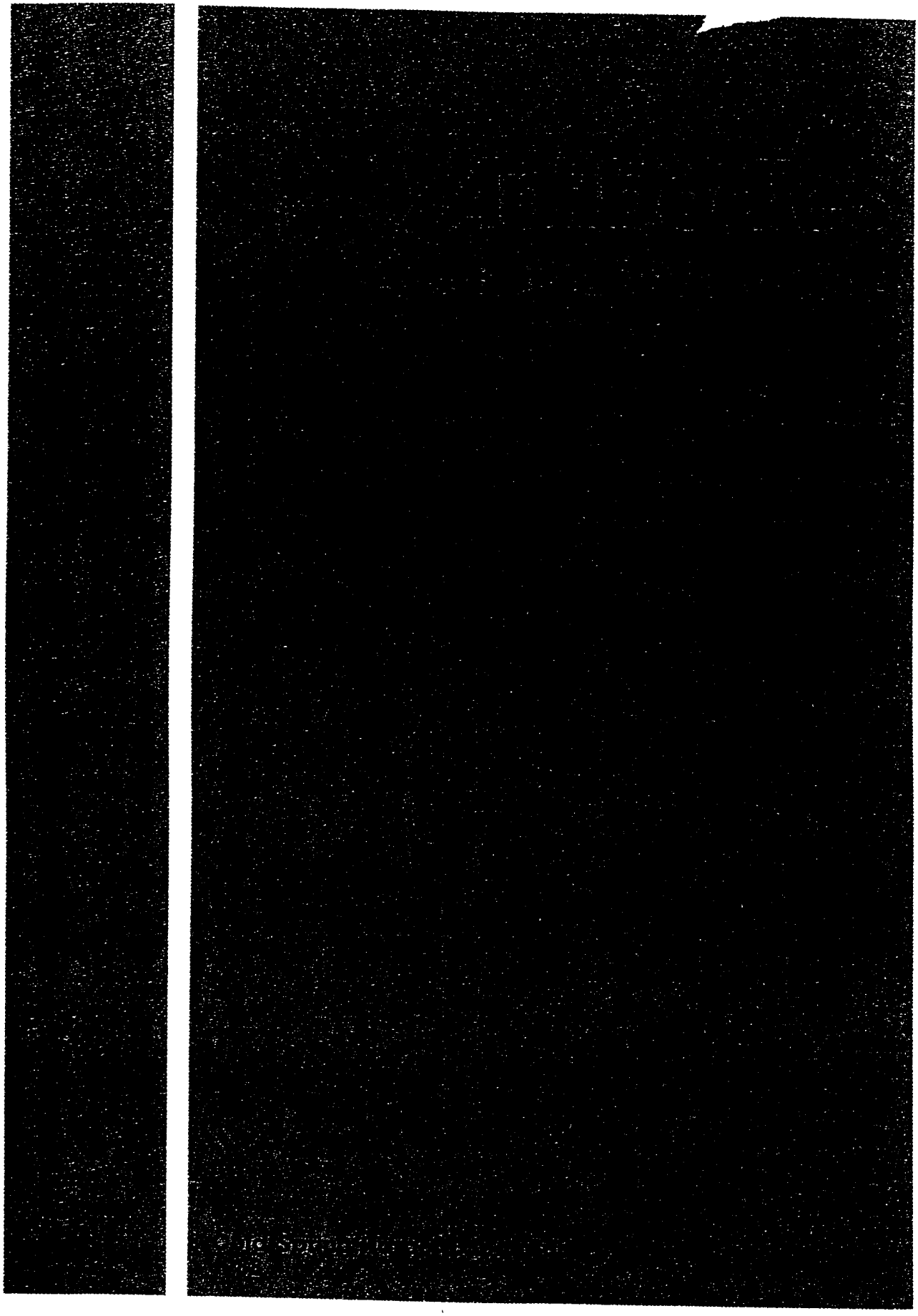

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A LABORATORY MANUAL

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14

IMMUNOASSAYS

Immunoassays are one of the most powerful of all immunochemical techniques. They employ a wide range of methods to detect and quantitate antigens or antibodies and to study the structure of antigens. With the appropriate assay, they can be remarkably quick and easy, yielding information that would be difficult to determine by other techniques. This chapter is divided into four sections, presenting (1) an overview of the different types of immunoassay, (2) a guide to help in choosing which assay to use, (3) the protocols themselves, and (4) the design of immunoassays.

TYPES OF IMMUNOASSAYS

There are many variations on the ways in which immunoassays can be performed, and they can be classified on the basis of many different criteria. In this manual, immunoassays are classified on the basis of methodology. Within each group, the principle and the order of the steps are similar. However, the variable that is being tested may change. For example, by changing certain key conditions, an assay can be altered to determine either antigen or antibody level. The steps are similar, but the assays yield different results. Although this organization provides a good structure to discuss technical strategies and problems, it is not always apparent which assays should be used for which applications, particularly to the novice. The section starting on p. 557 is designed to act as a guide to determine which assays may be appropriate for different applications.

As described here, the three classes of immunoassays are (1) the antibody capture assays, (2) the antigen capture assays, and (3) the two-antibody sandwich assays. The general procedures for these three types of assays are depicted in the cartoons in Figure 14.1, 14.2, and 14.3.

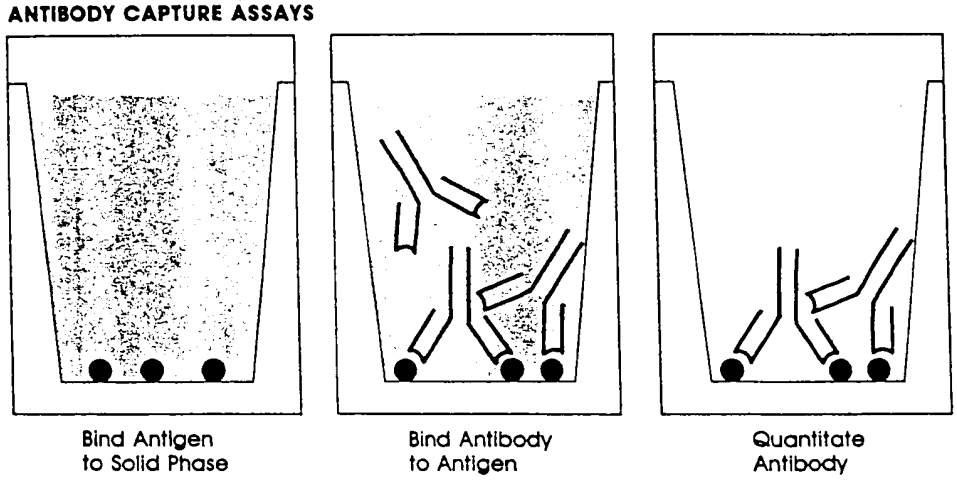


FIGURE 14.1
Antibody-capture assay.

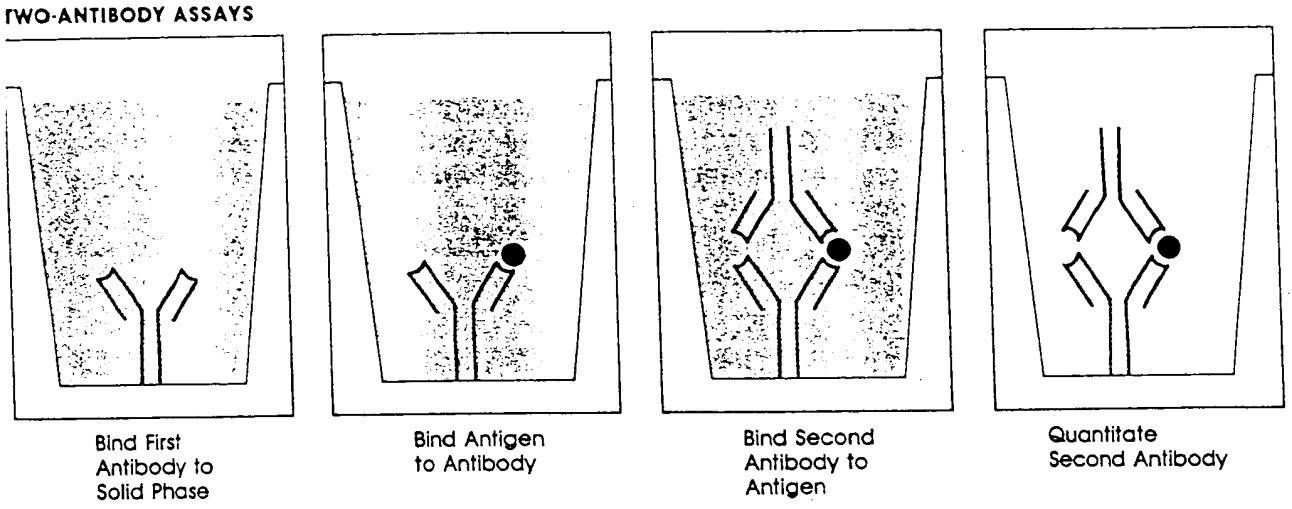


FIGURE 14.2
Two-antibody assays.

In an antibody capture assay, the antigen is attached to a solid support, and labeled antibody is allowed to bind. After washing, the assay is quantitated by measuring the amount of antibody retained on the solid support. In an antigen capture assay, the antibody is attached to a solid support, and labeled antigen is allowed to bind. The unbound proteins are removed by washing, and the assay is quantitated by measuring the amount of antigen that is bound. In a two-antibody sandwich assay, one antibody is bound to a solid support, and the antigen is allowed to bind to this first antibody. The assay is quantitated by measuring the amount of a labeled second antibody that can bind to the antigen. See Silman and Katchalski (1966) for an early review of the use of immobilized antibodies and antigens.

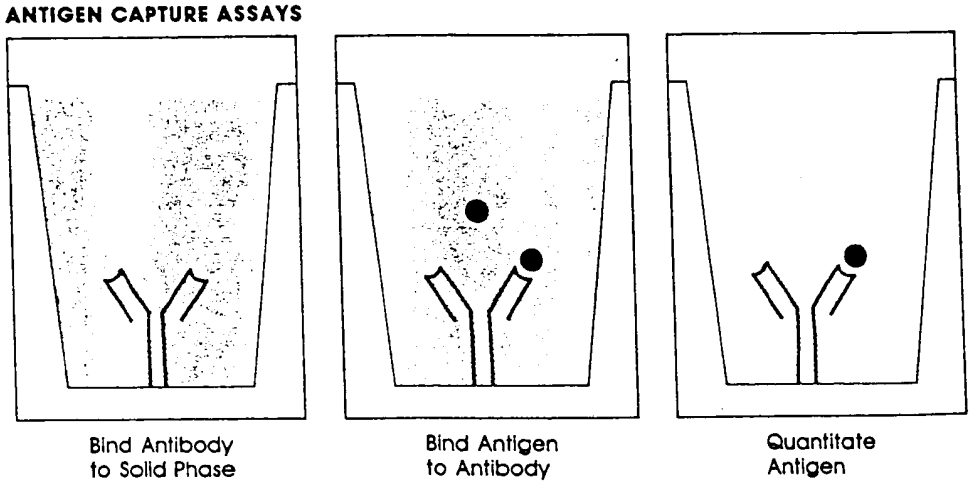


FIGURE 14.3
Antigen-capture assay.

DECIDING WHERE TO START

Any immunoassay can be performed with four variations; the assay can be done in antibody excess, in antigen excess, as an antibody competition, or as an antigen competition. Assays done in antibody excess or as antigen competitions are used to detect and quantitate antigens, while antigen excess or antibody competition assays are used to detect and quantitate antibodies. The four variations within the three classes yields a total of 12 possible immunoassays; however, not every combination leads to a useful immunoassay, and only six of the possibilities are commonly used. Representative protocols for each of these six assays are given on pp. 561–590.

In principle, the only factors that need to be considered in choosing the correct design of an immunoassay are the types of antibodies that are available (polyclonal antibodies, affinity-purified polyclonal antibodies, a single monoclonal antibody, or two or more monoclonal antibodies) and whether pure or impure antigen is available. Table 14.1 lists the possible choices based on these two variables and groups the assays in an order showing their general usefulness.

TABLE 14.1
Choosing an Assay Protocol

To measure	Type of antibody available	Type of antigen available	Assay choices[a]
Antigen Presence or Quantity	Polyclonal antibodies	Pure	1. Antigen capture (Ag competition), p. 586, 588 2. Antibody capture (Ag competition), p. 570
		Impure	1. Antibody capture (Ab excess), p. 574 Other assays possible, but need secondary technique
	Affinity-purified polyclonal antibodies	Pure	1. Two-antibody sandwich, p. 580 2. Antigen capture (Ag competition), pp. 586, 588 3. Antibody capture (Ag competition), p. 570
		Impure	1. Two-antibody sandwich, p. 580 2. Antibody capture (Ab excess), p. 574
	One monoclonal antibody	Pure	1. Antigen capture (Ag competition), pp. 586, 588 2. Antibody capture (Ag competition), p. 570
		Impure	1. Antibody capture (Ab excess), p. 574 Other assays possible, but may need secondary technique
	Two or more monoclonal antibodies	Pure	1. Two-antibody sandwich, p. 580 2. Antigen capture (Ag competition), pp. 586, 588 3. Antibody capture (Ag competition), p. 570
		Impure	1. Two-antibody sandwich, p. 580 2. Antibody capture (Ab excess), p. 574
Antibody Presence or Quantity[b]	Polyclonal antibodies	Pure	1. Antibody capture (Ag excess), p. 564
		Impure	Need secondary technique
	Affinity-purified polyclonal antibodies	Pure	1. Antibody capture (Ag excess), p. 564
		Impure	Need secondary technique
	One monoclonal antibody	Pure	1. Antibody capture (Ag excess), p. 564
		Impure	Need secondary technique
	Two or more monoclonal antibodies	Pure	1. Antibody capture (Ag excess), p. 564
		Impure	Need secondary technique

[a]The choice within each group are listed in order of suggested preference.
[b]For most assays used to measure antibody levels, the antibody is detected using an anti-immunoglobulin antibody. Thus, the antibody becomes the antigen.

Detecting and Quantitating Antigens

To detect and quantitate antigens, the most useful method is the two-antibody sandwich assay (p. 579). These assays are quick and reliable and can be used to determine the relative levels of most protein antigens. However, they require either two monoclonal antibodies that bind to independent sites on the antigen or affinity-purified polyclonal antibodies. An example of the two-antibody sandwich assay is found on p. 580.

If two monoclonal antibodies or affinity-purified polyclonal antibodies are not available, the next most useful assays for quantitating antigen are competition assays. For a competition assay, a sample of pure or nearly pure antigen is needed. There are two possible choices for assays using antigen competition. First, for an antigen capture assay (p. 585), the antigen is labeled and a constant amount of labeled antigen is mixed with the test solution which contains an unknown amount of the antigen. The solutions are then allowed to bind to a subsaturating amount of antibody bound to a solid phase. High levels of antigen in the test solution will reduce the amount of labeled antigen that can bind. Second, for an antibody capture assay (p. 563), a sample of pure or partially pure antigen is bound to the solid support. The antigen in the test solution is mixed with a preparation of labeled antibody and both are added to the solid support. High levels of antigen in the test solution will block the binding of the labeled antibody to the solid phase.

If pure or partially pure antigen is not available, the next most useful assay for antigen detection will be an antibody capture assay. Here the test solution is bound to a solid phase and a saturating amount of labeled antibody is used to detect the antigen. Two further factors must be considered. Because of their specificity, monoclonal antibodies or affinity-purified polyclonal antibodies will be more accurate than polyclonal antibodies when the antigen is rare. Similarly, as the antigen becomes more abundant, the specificity of the antibodies becomes less important.

Last, if rare antigens must be detected without the use of a pure sample of the antigen, the assay must be combined with a secondary technique. The secondary technique is used to distinguish the antigen from the background. Commonly used secondary techniques include immunoprecipitation, immunoblotting, and cell staining.

Detecting and Quantitating Antibodies

If pure or nearly pure antigen is available, antibody presence and level can be determined using a variation of the antibody capture assay (p. 563). Using this assay, purified or partially purified antigen is bound to a solid support. The test solution containing an unknown amount of antibody is allowed to bind to the antigen–matrix. The amount of antibody bound to the solid phase is determined by incubation with a labeled secondary reagent such as an anti-immunoglobulin antibody.

In many cases it is easiest to determine the presence or level of antibodies by considering them to be a special class of antigens and to use anti-immunoglobulin antibodies to detect them. In these cases, many of the assays discussed above can be used to detect these "antigens".

Secondary Techniques

When using impure sources of antigens for immunoassays, antibody detection may need to be supplemented with a secondary technique such as gel electrophoresis, precipitation, or immunoblotting. All monoclonal reagents have a certain background, and as the abundance of a particular antigen decreases relative to the other proteins, the ability of the antibodies to distinguish the antigen from the background is reduced. This is particularly true for polyclonal antibodies. When more specific antibodies are used, the amount of background decreases and less pure antigen sources can be used. In general, rare antigens will always need a secondary technique to differentiate the signal from the noise. Any technique that allows the antigen to be identified specifically among the background can be used as a secondary technique.

PROTOCOLS FOR IMMUNOASSAYS

The different techniques for immunoassays have been divided into three classes: (1) antibody capture assays, (2) antigen capture assays, and (3) two-antibody sandwich assays. Antibody capture assays are the most versatile. They can be used to determine both antigen or antibody levels and to compare antibody binding sites. Two-antibody sandwich assays are one of the best techniques for determining the presence and quantity of antigens. Antigen capture assays can be used for many types of studies, but are most useful for antigen detection and quantitation.

Two types of detection systems are commonly used for immunoassays. These are iodinated reagents and enzyme-labeled reagents. Assays that use iodinated reagents are easier to quantitate than enzyme-labeled reagents, while enzyme asssys will often yield a quicker result. The various detection methods are discussed in detail on p. 591.

Either iodinated or enzyme-labeled reagents can be used for direct or indirect methods. When using direct detection methods, the antibody or antigen is purified and labeled using one of the techniques described on p. 319. For indirect detection, a labeled secondary reagent that will bind specifically to an antibody is used. The secondary reagent can be labeled using one of the methods discussed on p. 319, but in most cases it is simpler to purchase these labeled reagents from commercial sources. A third variation that uses properties of both direct and indirect detection is the biotin–streptavidin system. Here, the antigen or antibody is purified and labeled with biotin (p. 340). The biotinylated reagent is detected by binding with streptavidin that has been labeled with iodine or an enzyme. Protocols for using all of these types of detection methods are given on p. 591.

SUMMARY

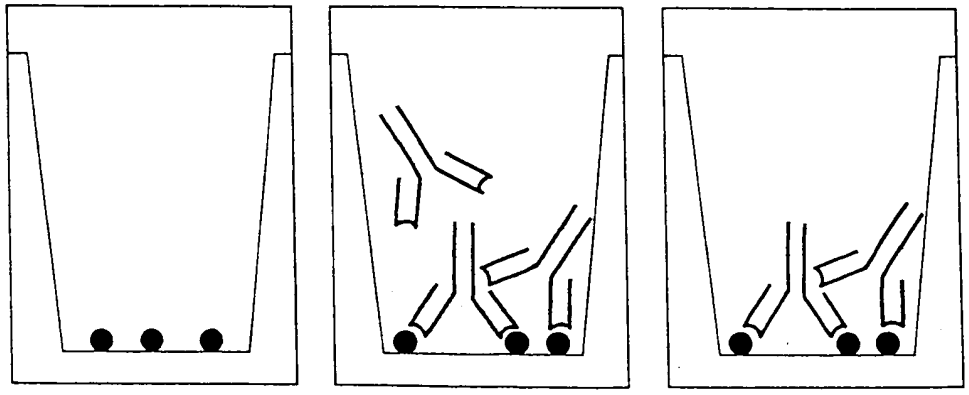
Antibody Capture Immunoassays

Detection limit is approximately 0.1–1.0 fmole, about 0.01–0.1 ng

- Rapid and easy
- Determines antigen presence and quantity
- Quantitative
- Sensitivity dependent on specific activity of antigen and avidity of antibody
- Needs high-avidity antibody

	Polyclonal Antibodies	Affinity-purified Polyclonal Antibodies	Monoclonal Antibodies	Pooled Monoclonal Antibodies
Signal strength	Fair	Excellent	Antibody dependent (poor to excellent)	Excellent
Specificity	Fair, but high background	Excellent	Excellent, but some cross-reactions	Excellent
Good Features	Availability	Specificity Signal strength	Specificity Unlimited supply Purity	Signal strength Specificity Unlimited supply Purity
Bad Features	Background Spurious activities Often need secondary techniques	Availability Limited supply	Need moderate to high avidity antibody	Availability

ANTIBODY CAPTURE ASSAYS



Bind Antigen to Solid Phase | Bind Antibody to Antigen | Quantitate Antibody

Antibody Capture Assays*

Antibody capture assays can be used to detect and quantitate antigens or antibodies and can be used to compare the epitopes recognized by different antibodies. The general protocol is simple; an unlabeled antigen is immobilized on a solid phase, and the antibody is allowed to bind to the immobilized antigen. The antibody can be labeled directly or can be detected by using a labeled secondary reagent that will specifically recognize the antibody. The amount of antibody that is bound determines the strength of the signal.

The three factors that will affect the sensitivity of a labeled antibody assay are (1) the amount of antigen that is bound to the solid phase, (2) the avidity of the antibody for the antigen, and (3) the type and number of labeled moieties used to label the antibody. First, the amount of antigen that is immobilized to the solid phase can be adjusted easily within the capacity of the chosen matrix by dilution or concentration of the antigen solution. Higher levels can only be achieved by changing the type of support. Polyvinylchloride (PVC) and nitrocellulose are the two most commonly used supports. PVC is normally used as a microtiter plate, while nitrocellulose is commonly used in sheets. Nitrocellulose will bind approximately 1000-fold more protein than PVC per unit of surface area. (See p. 605 for other possible matrices.) The second factor that will affect the sensitivity of an antibody capture assay is the avidity of the antibody for the immobilized antigen. Here, if other antibodies are available, they can be substituted for the original. If other antibodies are not available, the strength of the binding to the solid-phase antigen can often be increased by using a higher concentration of antigen. See Chapter 3 for a discussion of the advantages of bivalent binding and local concentration effects. Third, the type of label that is used and the specific activity of the labeled reagent will affect the sensitivity. Antibodies usually are labeled with either iodine (p. 324), enzymes (p. 342), or biotin (p. 340). The number of labeled molecules that are bound to the antibody can be varied to adjust the sensitivity of detection.

*Described originally by Miles and Hales (1968a,b).

ANTIBODY CAPTURE ASSAYS—DETECTING AND QUANTITATING ANTIBODIES USING ANTIGEN EXCESS ASSAYS

When labeled antibody assays are performed with excess antigen on the solid phase (i.e., enough to saturate all the available antibody), the presence and level of antibodies in a test solution can be measured. To perform the assay, a preparation of purified or partially purified antigen is bound to a solid substrate. The test solution or dilution of the test solution containing an unknown amount of antibody is added. Antibodies in the test solution are allowed to bind to the immobilized antigen, and unbound antibodies are removed by washing. The presence of the bound antibodies is detected using a labeled secondary reagent. This reagent could be labeled anti-immunoglobulin antibodies, protein A, or protein G (Chapter 15). Because the assay is performed using the indirect technique, the antibodies in the test solution never need to be purified. To quantitate the levels of antibody in the test solution, a titration of the test solution is performed, thus yielding the relative titers of the antibodies.

The degree of purity of the antigen solution needed for this assay will depend on the specificity of the antibodies in the test solution. Polyclonal sera will contain extraneous antibodies that will bind and score against some antigen preparations. Often these can be distinguished by titrating the sera. Because of their specificity, solutions of monoclonal antibodies can be tested using less pure sources of antigen.

1. Prior to the assay, prepare the secondary reagent. Suitable secondary reagents include anti-immunoglobulin antibodies, protein A, or protein G. These labeled reagents can be purchased from commercial sources with many different labels or can be prepared as described on p. 319.

2. The most widely used solid phase for these assays is a PVC microtiter plate. Cut the plate to the correct size for the number of assays.

3. Bind the pure or partially pure antigen to the bottom of the wells by adding 50 μl of antigen solution to each well. PVC will bind approximately 100 ng/well (300 ng/cm^2). If maximal binding is required, use at least 1 μg/well (20 μg/ml). Although this is well above the capacity of the PVC, the binding will occur more rapidly, and the binding solution can be saved and used again. If the antigen is rare or expensive, lower amounts of antigen can be added, but the sensitivity of the assay will decrease. If no other buffer is dictated, dilutions of antigen should be done in PBS. Avoid extraneous proteins and detergents which will compete with the antigen for binding to the plate.

4. Incubate for 2 hr at room temperature in a humid atmosphere.

5. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient. The antigen solution and washes can be removed by flicking the plate over a suitable waste container.

6. The remaining sites for protein binding on the PVC plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr to overnight in a humid atmosphere at room temperature.

7. Wash the plates twice with PBS.

8. Add 50 μl of the antibody test solution to each well. Incubate for 2 hr at room temperature in a humid atmosphere.

9. For quantitation, titrations of the test solution should be assayed (see p. 566). All antibody dilutions should be done in 3% BSA/PBS with 0.02% sodium azide.

10. Remove the unbound antibody by four washes with PBS.

11. Add the labeled secondary reagent. Use 50 μl/well. Incubate for 2 hr at room temperature.

12. For accurate quantitations, the amount of labeled secondary reagent must be in excess (see p. 566). All dilutions should be done in blocking buffer (3% BSA/PBS with 0.02% sodium azide). Azide should not be used with horseradish peroxidase detection systems.

13. Remove the unbound secondary reagent by washing four times with PBS.

Determine and quantitate the amount of labeled secondary reagent (p. 591).

NOTES

i. If the amount of antigen bound to the plate is too low to produce a strong signal, the solid support can be changed to nitrocellulose, which can bind approximately 1000 times more protein per surface area. Nitrocellulose can be purchased either as sheets or as a filter sealed to the bottom of microtiter wells.

ii. Any of blocking solutions on p. 496, Table 12.2, can be substituted for the 3% BSA solution.

iii. Antigen-coated plates can be stored after the blocking step (step 6) either in PBS with 0.02% sodium azide at 4°C for 1 week or indefinitely at −20°C after removing the blocking solution.

iv. For some applications or with some antibodies, the signal strength can be increased by using longer incubation times. For rapid semi-quantitative assays, the incubation times can be shortened to between 30 min and 1 hr. The blocking step must still be 2 hr or more.

MAKING THE ASSAY QUANTITATIVE

To compare the levels of antibody in different samples, prepare serial dilutions of each antibody test solution in blocking buffer. Perform the remainder of the assay as above. To determine the relative amounts of antibody, compare the midpoints of the titration curves. These assays will provide a relative measure of specific antibody concentration. To ensure that the assays are accurate, the amount of secondary labeled reagent that is used must be in excess. Nonsaturating amounts of labeled secondary reagent will yield good, but not excellent comparisons. The level of secondary antibody needed for saturation can be determined by titrating the secondary reagent, diluted in blocking buffer, on plates with saturating amounts of the primary antibody.

OTHER APPLICATIONS

- **Class/subclass determination** By changing the secondary reagent to class- or subclass-specific anti-immunoglobulin antibodies, the class or subclass of a particular monoclonal antibody can be determined easily.

- **Hybridoma screen** This technique is used as one of the most common methods for screening hybridoma tissue culture supernatants after fusion. See Chapter 6 for an example.

ANTIBODY CAPTURE ASSAYS—COMPARING ANTIBODY BINDING SITES USING AN ANTIBODY COMPETITION ASSAY

When antibody capture assays are performed using the antibody competition variation, the binding sites for two monoclonal antibodies can be compared. To use this assay, a sample of purified or partially purified antigen is bound to a solid support. Then two monoclonal antibodies are added, one labeled and one unlabeled. If the labeled antibody and the unlabeled antibody bind to separate and discrete sites on the antigen, the labeled antibody will bind to the same level whether or not the competing antibody is present. However, if the sites of interaction are identical or overlapping, the unlabeled antibody will compete, and the amount of labeled antibody bound to the antigen will be lowered. If the unlabeled antibody is present in excess, no labeled antibody will bind. With this assay, one monoclonal antibody will need to be purified and labeled, but none of the antibodies used as competitors need be purified.

Because only monoclonal antibodies are used in this technique, the antigen preparation need not be particularly pure. Samples containing as little as 1% pure antigen may be used, provided there are no cross-reacting proteins in the preparation.

1. Prior to the assay, purify and label each of the monoclonal antibodies that will be studied. Purification techniques are discussed in Chapter 8 and labeling techniques in Chapter 9.

2. The most widely used solid phase for this assay is a PVC microtiter plate. Cut the plate to the correct size for the number of assays.

3. Bind the standard antigen solution to the bottom of the wells by adding 50 μl of antigen solution to each well (20 μg/ml). PVC will bind approximately 100 ng/well (300 ng/cm^2). If maximal binding is required, use at least 1 μg/well. Although this is well above the capacity of the PVC, the binding will occur more rapidly, and the binding solution can be saved and used again. Avoid extraneous proteins, detergents, or other compounds that will lower the binding capacity of the PVC. If the choice of buffer is not dictated by experimental design, dilutions should be done in PBS.

   The amount of antigen bound to the wells should be high enough to produce an easily detectable antibody binding signal, but high concentrations also have some disadvantages. As the amount of antigen bound to the solid phase increases, the amount of competitor that is needed will increase. The amount of antigen should be titrated to the lowest needed to achieve a strong signal, thus increasing the sensitivity of the assay.

4. Incubate for 2 hr at room temperature in a humid atmosphere.

5. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient. The antigen solution or washes can be removed by flicking the plate over a suitable waste container.

6. The remaining sites for protein binding on the PVC plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr to overnight in a humid atmosphere at room temperature.

7. Wash the plate twice with PBS.

8. To each well add a mixture of two antibodies, one labeled and one unlabeled. Incubate for 2 hr at room temperature in a humid atmosphere.

   All antibody dilutions should be done in 3% BSA/PBS with 0.02% sodium azide. Buffers with azide should not be used when the detection system depends on horseradish peroxidase.

   To optimize this assay, the amount of labeled antibody should be titrated and used at a subsaturating level. These values can be determined in preliminary tests. For accurate quantitations, the amount of cold competitor should be titrated, and the midpoints of the competition curves compared.

9. Remove the unbound antibodies by four washes with PBS.

Determine and quantitate the amount of labeled antibody bound to the plate (p. 591).

NOTES

i. If the amount of antigen bound to the plate is too low to produce a strong signal, the solid support can be changed to nitrocellulose, which can bind approximately 1000 times more protein per surface area. Nitrocellulose can be purchased either as sheets or sealed to the bottom of microtiter wells. See p. 606 for special handling procedures.

ii. Any of blocking solutions on p. 496, Table 12.2, can be substituted for the 3% BSA solution.

iii. Antigen-coated plates can be stored after the blocking step (step 6) either in PBS with 0.02% sodium azide at 4°C for 1 week or indefinitely at −20°C after removing the blocking solution.

iv. For some applications or with some antibodies, the signal strength can be increased by using longer incubation times. For rapid semi-quantitative assays, the incubation times can be shortened to between 30 min and 1 hr. The blocking step must still be 2 hr or more.

MAKING THE ASSAY QUANTITATIVE

Competition between monoclonal antibodies for binding to an antigen can be quantitated in this assay. For this application, all monoclonal antibodies must be purified. A standard curve is established by titrating one monoclonal antibody against itself, that is, the same antibody is used for both the label and the competitor. The capacity of other unlabeled monoclonal antibodies to inhibit the binding of the labeled antibody to the plate is titrated. The results are plotted, and the concentrations necessary to achieve 50% inhibition of binding are compared.

OTHER APPLICATIONS

- **Screening hybridoma fusions for epitope-specific antibodies** This assay can be used to screen a hybridoma fusion for epitope-specific antibodies. The assay is best used as a complement to a preliminary screening procedure. In the preliminary screen, antibodies that bind to the antigen can be identified. Then, those monoclonal antibodies that bind antigen in a primary screen are tested in this antibody competition assay.

- **Quantitating antibodies specific for a particular epitope in polyclonal sera** If the competitor used in these assays is changed from an unlabeled monoclonal antibody to an unlabeled polyclonal antibody, the level of antibodies specific for that epitope in the polyclonal serum can be determined.

ANTIBODY CAPTURE ASSAYS—DETECTING AND QUANTITATING ANTIGENS USING ANTIBODY EXCESS ASSAYS

Using an antibody capture assay with the antigen competition variation, the presence and level of an antigen can be determined quickly. For this assay, purified or partially purified antigen is bound to the solid phase. A sample of the test solution, containing an unknown concentration of antigen, is added together with a labeled antibody specific for the antigen. Any antigen in the test solution will compete with the immobilized antigen for binding with the labeled antibody. After the unbound proteins are removed by washing, the assay is quantitated by the amount of labeled antibody that is bound to the solid phase. When high concentrations of antigen are found in the test solution, no labeled antibody will bind to the plate. When little or no antigen is present in the test solution, high amounts of labeled antibody will bind. To quantitate the levels of antigen, dilutions of the test solution are assayed. Comparing the titration curves for each solution will yield the relative levels of antigen.

The degree of purity needed for the immobilized antigen is determined by the specificity of the antibody. Polyclonal sera will contain extraneous antibodies that will bind and score against some impure antigen preparations. Often these can be distinguished by titrating the sera. Because of their specificity, solutions of monoclonal antibodies or affinity-purified polyclonal antibodies can be used with less pure sources of antigen.

One problem that may be encountered using this technique is that the labeled antibody may bind preferentially to the antigen on the plate. Quantitating this assay relies on a comparison of two avidities, the avidity of the antibody for the immobilized antigen and the avidity of the antibody for the antigen in solution. These avidities may not be the same. Because the antigen on the plate will be present in a high local concentration, the antibody may be able to bind bivalently to these antigens (see Chapter 3). These types of bivalent interactions cannot occur in solution when the antigen is monovalent. Not all immobilized antigens will promote bivalent binding, but many will. Problems generated by differences in avidity can be detected by comparing standard curves using known concentrations of antigens. If these assays give anomalous results, several steps can be used to help. First, try using lower concentrations of the antigen on the plate, thus decreasing the local concentration and decreasing the chances of bivalent binding. Second, premix the test solution with the labeled antibody and incubate for 30 min to 1 hr before adding to the plate. Third, the problem can be eliminated by using labeled Fab fragments (p. 626).

1. Prior to the assay, purify and label the primary antibody. The primary antibody can be labeled with iodine (p. 324), biotin (p. 340), or an enzyme (p. 342).

2. The most widely used solid phase for these assays is a polyvinylchloride (PVC) microtiter plate. Cut the plate to the correct size for the number of assays.

3. Prepare a standard solution of antigen. Avoid extraneous proteins and detergents.

4. Bind a sample of the standard antigen solution to the bottom of the wells by adding 50 μl of antigen solution to each well. PVC will bind approximately 100 ng/well (300 ng/cm^2). If maximal binding is required, use at least 1 μg/well (20 μg/ml). Although this is well above the capacity of the PVC, the binding will occur more rapidly, and the binding solution can be saved and used again. If the antigen is rare or expensive, lower concentrations can be used. Unless another buffer is dictated by experimental conditions, dilutions can be done in PBS.

   To optimize the assay, the amount of antigen on the plate should be adjusted to a level that will just bind all of the input labeled antibody but still give a satisfactory signal strength. See p. 573.

5. Incubate for 2 hr at room temperature in a humid atmosphere.

6. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient. The antigen solution or washes can be removed by flicking the plate over a suitable waste container.

7. The remaining sites for protein binding on the PVC plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr to overnight in a humid atmosphere at room temperature.

8. Wash the plates twice with PBS.

9. Add 50 μl of the antigen test solution together with the labeled antibody to each well. Incubate for 2 hr at room temperature in a humid atmosphere. These two solutions can be combined ahead of time and added together, or the antigen test solution can be added to the wells first, followed by the labeled antibody. However, the labeled antibody solution should not be added first.

   The amount of labeled antibody that is added will ultimately determine the signal strength; however, the antibody should not be added in excess over the binding capacity of the plate (see p. 573).

   All antibody and antigen dilutions should be done in 3% BSA/PBS with 0.02% sodium azide. Azide should not be included in the dilution buffer if horseradish peroxidase is used as the detection reagent.

10. Remove the unbound antibody and antigen by four washes with PBS.

Determine and quantitate the amount of labeled antibody bound to the plate (p. 591).

NOTES

i. If the amount of antigen bound to the plate is too low to produce a strong signal, the solid phase can be changed to nitrocellulose, which can bind approximately 1000 times more protein per surface area. Nitrocellulose can be purchased either as sheets or sealed to the bottom of microtiter wells. See p. 606 for special handling procedures.

ii. Any of blocking solutions on p. 496 can be substituted for the 3% BSA solution.

iii. Antigen-coated plates can be stored after the blocking step (step 7) either in PBS with 0.02% sodium azide at 4°C for 1 week or indefinitely at −20°C after removing the blocking solution.

iv. This assay may also be performed using an indirect detection method. The primary antibody is not labeled, but the remainder of the assay is performed as above. After the final wash (step 10), add a labeled secondary reagent. Incubate for 2 hr at room temperature, wash, and quantitate.

MAKING THE ASSAY QUANTITATIVE

To compare the levels of antigen in different samples, prepare serial dilutions of each antigen test solution in blocking buffer. Perform the remainder of the assay as above. To determine the relative amounts of antigen, compare the midpoints of the titration curves. To determine the absolute amount of antigen, compare these values with those obtained using known amounts of pure antigen in a standard curve.

For maximum sensitivity, first titrate the amount of standard antigen solution needed to coat the plate versus a fixed, high concentration of labeled antibody. Plot the values and select the lowest level that will yield a strong signal. Next, using plates coated with this amount of standard antigen solution, titrate the labeled antibody. Plot these values and select a level of antibody that is within the linear portion of the curve. Maximum range is obtained by choosing a point near the saturation level.

ANTIBODY CAPTURE ASSAYS—DETECTING AND QUANTITATING ANTIGENS USING ANTIGEN COMPETITION ASSAYS

An antibody capture assay performed in antibody excess can be used to determine the presence and level of antigens in test solutions. The test solution is allowed to bind directly to a solid phase, and any unbound proteins are removed by washing. Then an antibody specific for the antigen is added and allowed to bind. After unbound antibodies are removed by washing, the amount of antibody adhering to the solid phase is determined by using a labeled secondary reagent. This secondary reagent could be a labeled anti-immunoglobulin antibody, protein A, or protein G. These assays cannot be used if the antigen being studied is a rare component of the test solution. In this case, the specific binding is obscured by the background. A secondary technique such as immunoblotting may be particularly valuable to help distinguish the specific signal from the background when rare antigens are being studied.

The specificity of the antibody will determine the degree of purity needed to detect the antigen. Polyclonal sera will contain extraneous antibodies that will bind and score against some antigen preparations. Often these can be distinguished by titrating the sera. Because of their specificity, monoclonal antibodies or affinity-purified polyclonal antibodies can be used to analyze less pure sources of antigen.

Making these assays quantitative is difficult for samples that contain widely varying amounts of total protein. In these cases, the total amount of protein added to the solid phase must be standardized.

1. Prior to the assay, prepare the secondary reagent. Suitable secondary reagents include anti-immunoglobulin antibodies, protein A, or protein G. These reagents can be purchased from commercial sources with many different labels or they can be prepared as described on p. 319.

2. Nitrocellulose is the most suitable support for these assays, because its high binding capacity permits the detection of less abundant antigens in the sample. If using the nitrocellulose for a sheet assay, mark the nitrocellulose into 3-mm squares using a soft lead pencil. Cut the sheet to the proper size for the number of assays to be performed. If using the nitrocellulose paper in a dot blot apparatus, cut to the dimensions of the apparatus.

3. Antigens should be prepared in neutral pH buffers without additional proteins. If no other buffers are dictated by the experimental design, use PBS. Detergents should be avoided if possible. If they must be used, test the effects of different possible detergents on antigen binding to the nitrocellulose. For sheet assays, the antigen test solutions are added to the center of individual squares (1 μl/spot). Incubate for 30 min at room temperature in a humid atmosphere. For dot blot assays, pre-wet the sheet by floating on water for 5 min. Fit the sheet into the apparatus and apply the antigen test solution to the region of nitrocellulose that is exposed. Use 30 μl/well. Incubate for 2 hr at room temperature in a humid atmosphere.

4. If using a dot blot apparatus, remove the nitrocellulose sheet. Wash the nitrocellulose with two changes of PBS.

5. Block the remaining sites for protein binding on the nitrocellulose by incubating the sheet in 3% BSA/PBS with 0.02% sodium azide for at least 2 hr at room temperature.

6. Wash the nitrocellulose sheet twice in PBS.

7. Add primary antibody at a suitable dilution to the entire sheet. Use 1 ml/cm^2. Incubate with agitation for 2 hr at room temperature. The amount of antibody to be added can be determined in preliminary tests. Higher amounts will increase the sensitivity and extend the range of the assay, but also will increase the possibility of detecting nonspecific antigens.

   All antibody dilutions should be done in 3% BSA/PBS with 0.02% sodium azide.

8. Remove the unbound antibody by four washes with PBS.

9. Add the labeled secondary reagent. Use 1 ml/cm^2. The amount of labeled secondary reagent should be determined in preliminary tests. For accurate comparisons, the secondary reagent should be used in excess. For routine comparisons, the level of secondary reagent should be adjusted to produce a easily detected signal. All dilutions should be done in blocking buffer (3% BSA/PBS with 0.02% sodium azide).

   Incubate with agitation for 2 hr at room temperature.

10. Remove the unbound antibody by washing four times with PBS for 5 min each.

Determine and quantitate the amount of labeled secondary reagent bound to the sheet (p. 591).

NOTES

i. Any of blocking solutions on p. 496 can be substituted for the 3% BSA solution.

ii. If the antigen is impure, then a solid-phase substrate of higher capacity than nitrocellulose may be required to generate adequate signal strength. For this purpose, diazotized paper is useful as it can bind approximately 100 times more protein than nitrocellulose. With this variation, background problems caused by nonspecific binding or spurious cross-reactions may be severe.

iii. Directly labeled primary antibodies can be used in this test.

MAKING THE ASSAY QUANTITATIVE

To compare the levels of antigen in different samples, prepare serial dilutions of each antigen test solution in PBS. Perform the remainder of the assay as above. To determine the relative amounts of antigen, compare the midpoints of the titration curves. To determine the absolute amount of antigen, compare these values with those obtained using known amounts of pure antigen in a standard curve. To ensure that the assays are accurate, the amount of primary unlabeled antibody and secondary labeled reagent must be in excess. This can be determined by titrating the reagents, diluted in blocking buffer, on sheets coated with saturating amounts of the antigen.

OTHER APPLICATIONS

- **Column profiles** Assays of this design are extremely useful where rapid estimates of antigen presence and quantity are required for large sample numbers. A good example of this application is the detection of antigen-positive fractions from conventional chromatography columns or gradients.

SUMMARY

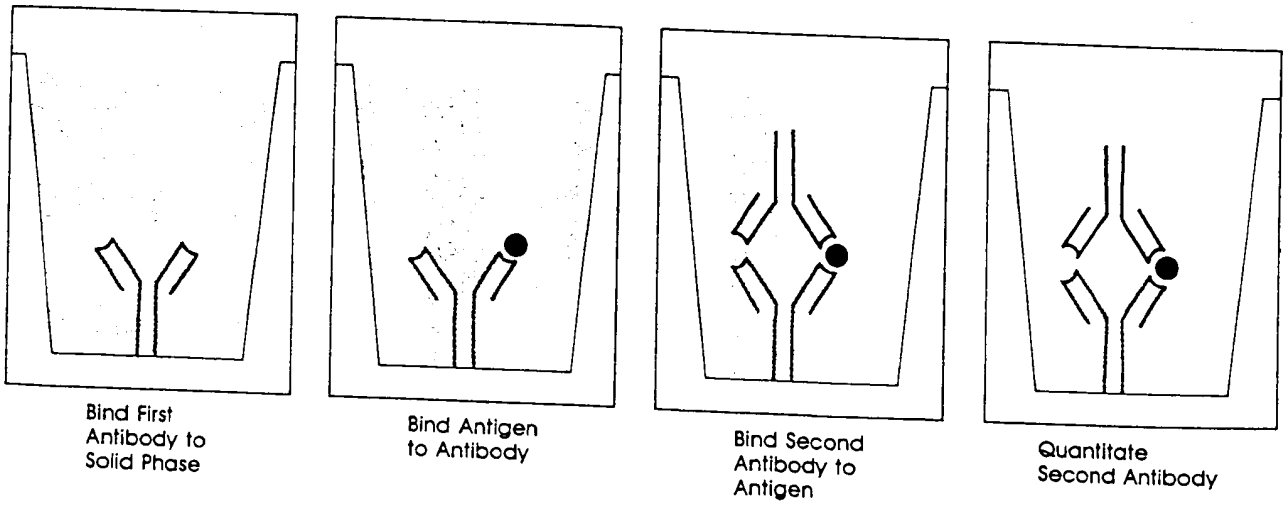
Two-antibody Sandwich Immunoassays

Detection limit is approximately 0.1–1.0 fmole, about 0.01–0.1 ng

- Rapid and easy
- Detects antigen presence and quantity
- Quantitative
- Sensitivity dependent on specific activity of antibody and affinity of antibodies
- Needs affinity-purified polyclonal antibodies or two monoclonal antibodies with high affinity

	Polyclonal Antibodies	Affinity-purified Polyclonal Antibodies	Monoclonal Antibody	Multiple Monclonal Antibodies
Signal Strength	—	Excellent	—	Excellent
Specificity	—	Excellent	—	Excellent
Good Features	—	Multivalent	—	Unlimited supply
Bad Features	Cannot use polyclonal antibodies	Availability	Cannot use single antibody	Availability Need two antibodies that bind distinct epitopes

TWO-ANTIBODY ASSAYS



Two-Antibody Sandwich Assays

One of the most useful of the immunoassays is the two-antibody sandwich technique. These assays are used primarily to determine the antigen concentration in unknown samples. Two-antibody assays are quick and accurate, and if a source of pure antigen is available, the assays can be used to determine the absolute amounts of antigen in unknown samples. The assay requires two antibodies that bind to non-overlapping epitopes on the antigen. Either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies can be used.

To use the two-antibody assay, one antibody is purified and bound to a solid phase, and the antigen in a test solution is allowed to bind. Unbound proteins are removed by washing, and the labeled second antibody is allowed to bind to the antigen. After washing, the assay is quantitated by measuring the amount of labeled second antibody that is bound to the matrix. The major advantages of this technique are that the antigen does not need to be purified prior to use and that the assays are very specific. The major disadvantage is that not all antibodies can be used.

The sensitivity of two-antibody assays is dependent on four factors: (1) the number of molecules of the first antibody that are bound to the solid phase, (2) the avidity of the first antibody for the antigen, (3) the avidity of the second antibody for the antigen, and (4) the specific activity of the labeled second antibody. First, the amount of the first antibody that is bound to the solid phase can be adjusted easily within the capacity of the chosen matrix by dilution or concentration of the antibody solution. Higher capacities can be achieved by changing the type of support. Polyvinylchloride is the most commonly used matrix, but several solid supports can be substituted to increase the linear range of the assay. The most useful alternative support is nitrocellulose, which binds approximately 1000 times more protein per surface area. (See p. 605 for other possible matrices.) The second and third factors that affect the sensitivity of the assays are the avidity of the antibodies for the antigen. These can only be altered by substitution with other antibodies. The fourth factor is the number and type of labeled moeities on the second antibody. Antibodies can be labeled conveniently with iodine, enzymes, or biotin. The choice of label is discussed on p. 591.

DETECTING AND QUANTITATING ANTIGENS USING THE TWO-ANTIBODY SANDWICH ASSAY*

1. Prior to the assay, both antibody preparations need to be purified (p. 288) and one needs to be labeled (p. 319). For monoclonal antibodies, choosing which antibody to label is determined empirically. When setting up the assay, label each antibody preparation independently, and try both combinations of solid-phase and labeled antibody to determine which is best. When using affinity-purified antibodies, the same antibody preparation will be used for both sides of the assay.

2. For most applications, a PVC microtiter plate is best. Cut the plate to the correct size for the number of assays.

3. Bind the unlabeled antibody to the bottom of each well by adding approximately 50 μl of antibody solution to each well (20 μg/ml in PBS). PVC will bind approximately 100 ng/well (300 ng/cm^2). The amount of antibody to be used will depend on the individual assay, but if maximal binding is required, use at least 1 μg/well. Although this is well above the capacity of the well, the binding will occur more rapidly, and the binding solution can be saved and used again. In most cases, saturating amounts of the first antibody will increase the sensitivity of the assays and will not have any detrimental effects.

   Incubate for 2 hr at room temperature in a humid atmosphere.

4. Wash the wells twice with PBS. A 500-ml squirt bottle is convenient. The antibody solution or washes can be removed by flicking the plate over a suitable waste container.

5. The remaining sites for protein binding on the polyvinylchloride plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr to overnight in a humid atmosphere at room temperature.

6. Wash the wells twice with PBS.

7. Add 50 μl of the antigen solution to the wells. For quantitation, the antigen solution should be titrated. All dilutions should be done in the blocking buffer (3% BSA/PBS with 0.02% sodium azide). Incubate for at least 2 hr at room temperature in a humid atmosphere.

*Ling and Overby (1972); Belanger et al. (1973); Maiolini and Masseyeff (1975).

8. Wash the plate four times with PBS.

9. Add the labeled second antibody. The amount of labeled antibody to be added can be determined in preliminary experiments. For accurate quantitation, the second antibody should be used in excess. All dilutions of the labeled antibody should be done in blocking buffer (3% BSA/PBS with 0.02% sodium azide).

10. Incubate for 2 hr or more at room temperature in a humid atmosphere.

11. Wash with several changes of PBS.

Quantitate the amount of bound labeled antibody using the proper detection method (p. 591).

NOTES

i. Any of blocking solutions on p. 496 can be substituted for the 3% BSA solution.

ii. Antibody-coated plates can be stored after the blocking step (step 5) either in PBS with 0.02% sodium azide at 4°C for 1 week or indefinitely at −20°C after removing the blocking solution.

iii. For some applications or with some antibodies, the signal strength can be increased by using longer incubation times. For rapid semi-quantitative assays, the incubation times can be shortened to between 30 min and 1 hr. The blocking step must still be 2 hr or more.

MAKING THE ASSAY QUANTITATIVE

The amount of second antibody should be in excess to ensure that the assay is quantitative. The correct amount to add can be determined by titrating the amount of labeled second antibody on antigen-saturated plates. To compare the relative amounts of antigen in different test samples, prepare serial dilutions of each sample in blocking buffer. Perform the assays as described above. Plot and compare the midpoints of each titration curve. To determine absolute amounts of antigen, compare these plots to a standard curve obtained using known amounts of pure antigen.

OTHER APPLICATIONS

- **Epitope comparisons** This basic assay design can be adapted to perform antibody competition experiments. This is particularly useful when purified antigen is not available to establish the antibody capture/competition assay described on p. 567. The antibodies, whose binding sites are to be compared, are purified and labeled. A third antibody is used to coat the wells. Each labeled antibody is tested for its ability to bind to the antigen immobilized through this third antibody. The test can proceed if both labeled antibodies bind efficiently. For the test, varying amounts of the unlabeled, test monoclonal antibody are added to the antigen-coated wells, followed by a fixed, subsaturating amount of the labeled antibody. If the unlabeled antibody and the labeled antibody bind to overlapping sites on the antigen, then the amount of label bound to the solid phase will be reduced. If the unlabeled antibody and the labeled antibody bind to nonoverlapping sites, there will be no reduction in the binding of labeled antibody. By varying the antibody used to coat the well, many different areas of the antigen can be studied in this way, and a steric inhibition map of the antigen developed.

- **Studying protein complexes** Two-antibody sandwich assays can be used to study a number of aspects of protein complexes. If antibodies are available to different components of a heteropolymer, a two-antibody assay can be designed to test for the presence of the complex. An antigen competition assay can be designed to measure the quantity of either component of the complex.

  Using a variation of these assays, monoclonal antibodies can be used to test whether a given antigen is multimeric. If the same monoclonal antibody is used for both the solid phase and the label, monomeric antigens cannot be detected. Such combinations, however, may detect multimeric forms of the antigen. Be cautious; in these assays negative results may be generated both by multimeric antigens held in unfavorable steric positions as well as by monomeric antigens.

SUMMARY

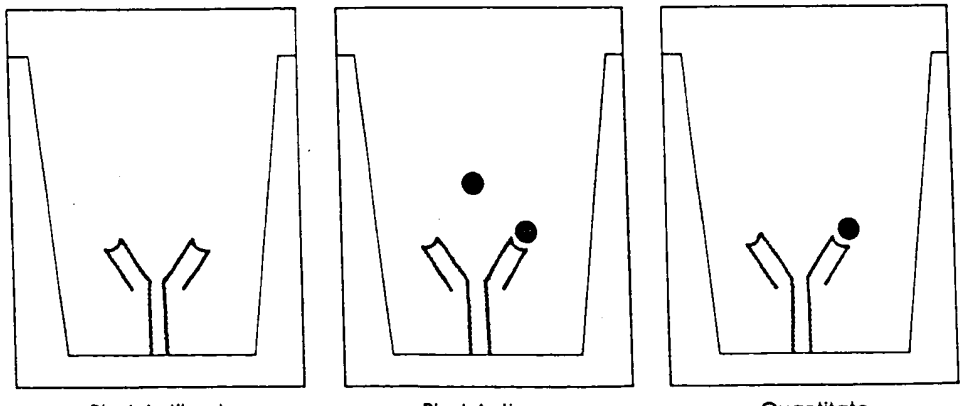
Antigen Capture Immunoassays

Detection limit is approximately 0.1–1.0 fmole, about 0.01–0.1 ng

- Rapid and easy
- Determines antigen presence and quantity
- Quantitative
- Sensitivity dependent on specific activity of antigen and avidity of antibody
- Needs high-avidity antibody

	Polyclonal Antibodies	Affinity-purified Polyclonal Antibodies	Monoclonal Antibodies	Pooled Monoclonal Antibodies
Signal Strength	Excellent	Excellent	Antibody dependent (poor to excellent)	Excellent
Specificity	Usually good, but some background	Excellent	Excellent, but some cross-reactions	Excellent
Good Features	Stable multivalent interactions	Stable, multivalent interactions	Specificity Unlimited supply	Stable, multivalent interactions Specificity Unlimited supply
Bad Features	Nonrenewable Background	Availability	Need high affinity	Availability

ANTIGEN CAPTURE ASSAYS



Antigen Capture Assays*

Antigen capture assays are used primarily to detect and quantitate antigens. In both the variations discussed here, the amount of antigen in the test solution is determined using a competition between labeled and unlabeled antigen. Unlabeled antibody is bound to the solid phase either directly or through an intermediate protein, such as an anti-immunoglobulin antibody. The antigen is purified and labeled. A sample of the labeled antigen is mixed with the test solution containing an unknown amount of antigen, and the mixture is added to the bound antibody. The antigen in the test solution will compete with the labeled antigen for binding to the antibody–matrix. Unbound proteins are removed by washing, and the amount of labeled antigen bound to the matrix is measured. If the unknown solution contains a high concentration of antigen, it will compete effectively with the labeled antigen, and little or none of the labeled antigen will bind to the antibody. To quantitate the levels of antigen, dilutions of the test solution are performed and assayed. Comparing the titration curves for each solution will yield the relative levels of antigen.

The sensitivity of the labeled antigen assay will depend on three factors: (1) the number of antibodies that are bound to the solid phase, (2) the avidity of the antibody for the antigen, and (3) the specific activity of the labeled antigen. First, the number of antibodies that are bound to the solid phase can be adjusted within the capacity of the matrix by diluting or concentrating the antibody solution. Higher levels can be achieved by changing the type of solid-phase matrix that is used. Second, the avidity of the antibody for the antigen will affect the sensitivity of the assay, but can only be altered by changing the antibody source. Third, the choice of label for the antigen is discussed on p. 591.

The degree of purity needed for the labeled antigen will be determined by the specificity of the antibody. Polyclonal sera will contain extraneous antibodies that will bind and score against some antigen preparations. Often these can be distinguished by titrating the sera. Because of their specificity, solutions of monoclonal antibodies can be used with less pure sources of antigen.

*Originally developed by Yalow and colleagues. See Berson et al. (1956); Yalow and Berson (1959) for the concepts, Weiler et al. (1960) for competition assays, Wide and Porath (1966) for the use of a solid phase.

DETECTING AND QUANTITATING ANTIGENS USING COMPETITION ASSAYS—VARIATION I, MICROTITER PLATES

This assay is given in two variations, here set up for microtiter plates and in the next technique for *Staphylococcus aureus* binding. The microtiter plate technique is useful when the antibody is easily purified and when there are a large number of antigen samples to test. The *S. aureus* assay is best when a small number of samples will be tested. The *S. aureus* variation also does not require that the antibody be purified.

1. Prior to the assay, label the antigen. Purified or partially purified antigen can be labeled with iodine (p. 324), enzymes (p. 342), or biotin (p. 340). Abundant antigens can also be labeled metabolically by growing cells in the presence of a radioactive precursor (p. 358).

2. Prior to the assay, purify the antibody as described on p. 288.

3. Bind the unlabeled antibody to the bottom of each well by adding approximately 50 μl of antibody solution to each well (20 μg/ml in PBS). Polyvinylchloride (PVC) will bind approximately 100 ng/well (300 ng/cm^2). The amount of antibody to be used will depend on the individual assay, but if maximal binding is required, use at least 1 μg/well. Although this is well above the capacity of the well, the binding will occur more rapidly, and the binding solution can be saved and used again.

   Maximal binding may not always be desirable in these assays. Higher amounts of antibody bound to the solid phase increase the level of antigen needed for competition to be detected. See p. 587 for a discussion of the parameters that need to be checked to make this assay quantitative.

   Incubate for 2 hr at room temperature in a humid atmosphere.

4. Wash the wells twice with PBS. A 500-ml squirt bottle is convenient. The antibody solution or washes can be removed by flicking the plate over a suitable waste container.

5. The remaining sites for protein binding on the polyvinylchloride plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr to overnight in a humid atmosphere at room temperature.

6. Wash the wells twice with PBS.

7. Add 50 μl of the antigen test solution along with the standard labeled antigen solution to the wells. Either add the test solution first, followed immediately by the labeled solution or mix in a separate plate and transfer together.

   The amount of labeled antigen to be added should be sufficient to generate a strong signal, but should not greatly exceed the capacity of the antibody matrix (see p. 605). For accurate quantitation the test solution should be assayed over a number of dilutions.

   All dilutions should be done in the blocking buffer (3% BSA/PBS with 0.02% sodium azide).

   Incubate for 2 hr at room temperature in a humid atmosphere.

8. Wash the plate four times with PBS.

Determine the amount of labeled antigen bound (p. 591).

NOTES

i. If the amount of antibody bound to the plate is too low to produce a strong signal, the solid support can be changed to nitrocellulose, which can bind approximately 1000 times more protein per surface area. Nitrocellulose can be purchased either as sheets or sealed to the bottom of microtiter wells. See p. 606 for special handling procedures.

ii. Any of blocking solutions on p. 496 can be substituted for the 3% BSA solution.

iii. Antibody-coated plates can be stored after the blocking step (step 5) either in PBS with 0.02% sodium azide at 4°C for 1 week or indefinitely at −20°C after removing the blocking solution.

MAKING THE ASSAY QUANTITATIVE

To compare the levels of unlabeled antigen in different samples, prepare serial dilutions of each antigen test solution in blocking buffer. Perform the remainder of the assay as above. To determine the relative amounts of antigen, compare the mid points of the titration curves. To determine the absolute amount of antigen, compare these values with those obtained using known amounts of pure unlabeled antigen in a standard curve.

For maximum sensitivity, first titrate the amount of purified antibody bound to the plate versus a saturating amount of labeled antigen solution without the addition of competing unlabeled antigen. Plot the values and select the lowest level that will yield a strong signal. Next, using plates coated with this amount of antibody, titrate the labeled antigen. Plot the percentage of input antigen bound versus the total antigen input. Select a level of antigen that is within the linear portion of the curve. Maximum range is obtained by choosing a point near the saturation level.

DETECTING AND QUANTITATING ANTIGENS USING COMPETITION ASSAYS—VARIATION II, S. AUREUS

In this version of the antigen capture assay, the solid-phase matrix is provided by adding *Staphylococcus aureus*. These killed bacteria have a high concentration of protein A on their surface that binds the antibody in the solution. The *S. aureus* assay is best when a small number of samples will be tested and also does not require that the antibody be purified.

1. Prior to the assay, label the antigen with iodine (p. 324). Other labels are not as useful with this assay design, because the results are quantitated from 1.5-ml conical tubes and iodine is easy to detect by counting the entire tube in a gamma-counter.

2. Mix a 50-μl sample of labeled antigen with a 50-μl sample of the unlabeled test solution in a 1.5-ml conical tube.

   The amount of labeled antigen to be added should be sufficient to generate a strong signal, but should not greatly exceed the capacity of the antibody that will be added (see p. 589). For accurate quantitation, a series of dilutions of test samples should be assayed. All dilutions should be done in 3% BSA/PBS with 0.02% sodium azide.

3. Add 100 μl of the antibody solution to the 1.5-ml conical tube. The concentration of antibody should be subsaturating to detect the competition. The preliminary tests needed to adjust the amount of antibody to add are discussed on p. 589. All dilutions should be done in 3% BSA/PBS with 0.02% sodium azide.

   Incubate at room temperature for 2 hr.

4. If the specific antibody binds protein A tightly (see p. 616), add 50 μl of a 10% suspension of fixed *S. aureus* cells (prewashed twice in PBS, see p. 620).

   If the particular antibody does not bind protein A, add 50 μl of a dilution of an anti-immunoglobulin antibody raised in a species whose IgG bind protein A (e.g., rabbits). Incubate for 1 hr, and add 50 μl of a 10% suspension of fixed *S. aureus* cells (prewashed twice in PBS, see p. 620). For complete binding of the first antibody to the solid phase, the anti-immunoglobulin antibody must be in excess. This should be titrated to determine the correct value; however, 5 μl of a high-titer rabbit sera will bind approximately 1 μg of mouse antibody. All dilutions should be done in 3% BSA/PBS with 0.02% sodium azide.

5. Incubate for 15 min at room temperature.

6. Add 1.0 ml of PBS containing 1% NP-40 to each tube. Centrifuge for 3 min.

7. Aspirate the supernatant to remove unbound antigen.

8. Determine the amount of bound antigen in the pellet by counting the entire tube in a gamma counter.

MAKING THE ASSAY QUANTITATIVE

To compare the levels of unlabeled antigen in different samples, prepare serial dilutions of each antigen test solution in blocking buffer. Perform the remainder of the assay as above. To determine the relative amounts of antigen, compare the mid points of the titration curves. To determine the absolute amount of antigen, compare these values with those obtained using known amounts of pure unlabeled antigen in a standard curve.

For maximum sensitivity, first titrate the amount of antibody versus a saturating amount of labeled antigen solution without the addition of unlabeled antigen. Plot the values and select the lowest level that will yield a strong signal (approximately 10,000–50,000 cpm). Next, using this amount of antibody, titrate the labeled antigen. Plot the percentage of input antigen bound versus the total antigen input. Select a level of antigen that is within the linear portion of the curve. Maximum range is obtained by choosing a point near the saturation level.

Epitope Mapping

For some purposes it is necessary to determine whether individual monoclonal antibodies raised against the same antigen bind to identical or overlapping epitopes. Three methods commonly are used to test whether antibodies recognize similar sites on protein antigens.

- **Steric competition** To set up a steric competition immunoassay, one of the monoclonal antibodies is purified and labeled. The capacity of other monoclonal antibody preparations to block the binding of the labeled antibody to the antigen is tested. Two methods for these types of assay are described on pp. 567 and 583.

- **Linear maps** If a DNA clone is available for a protein antigen, deletion mutations can be assayed for the production of fragments that can be recognized by an antibody. In practice, this method can only localize the binding site to a small region if the monoclonal antibody recognizes an epitope that is formed by a contiguous stretch of the primary sequence of the protein. If the antibody binds to an epitope formed by a secondary or tertiary structure, this technique will only identify a relatively large region containing the epitope. When locating the boundaries of an epitope using this method, only results that produce a positive binding should be used to localize the binding site. Lack of binding can be caused by reasons other than the loss of the epitope, such as steric interference, long-range conformation changes, or rapid degradation.

- **Random cloning of epitopes in bacteria** If the monoclonal antibodies can recognize the antigen synthesized in bacteria, a simple and rapid method to determine the location of the epitope is randomly to clone small fragments of the coding region into an expression vector and screen for binding of the antibody to the bacterially expressed protein. Random fragments can be generated using sonication (Deininger 1983) or partial DNase I digestion (Anderson 1981) and cloned by blunt-end ligation into a suitable site of a fusion protein vector. Expression of the epitope can then be determined by screening lifts for antibody binding (see Helfman et al. 1983 or Young and Davis 1983a,b for appropriate antibody screening techniques or pp. 691 and 692). If expression is combined with DNA sequencing, an accurate location of the epitope can be determined.

Detection

All immunoassays rely on labeled antigens, antibodies, or secondary reagents for detection. These proteins can be labeled with radioactive compounds, enzymes, biotin, or fluorochromes. Of these, radioactive labeling can be used for almost all types of assays and with most variations. It is easy to quantitate and simple to detect. Enzyme-conjugated labels are particularly useful when radioactivity must be avoided or when quick results are helpful. They yield semi-quantitative results with no adjustments, but are more troublesome to use for exact quantitation. Although it is widely argued, neither is remarkably more sensitive than the other. In the future, however, it is clear that the use of new and more sensitive enzyme substrates will make these assays more valuable.

Biotin-coupled reagents usually are detected with labeled streptavidin. Streptavidin binds tightly and quickly to biotin and can be labeled with iodine or enzymes.

Of the methods used to label proteins, fluorochromes have the fewest applications for immunoassays. Although they can be very sensitive, they require rather expensive equipment to use and, unlike radiometric or enzymatic detection, no alternative methods can be used to locate and quantitate positives. In the foreseeable future, their major use will continue to be in clinical and large-volume laboratories where numerous samples need to be processed.

Iodine-Labeled Antigens, Antibodies, or Secondary Reagents

Iodine-labeled reagents can be detected either by autoradiography or by direct counting. For autoradiographic detection the sample should be placed in direct contact with the film and placed at −70°C with an intensifying screen. Results can be quantitated at a crude level by simple visual examination of the exposed film and more quantitatively by densitometric tracing. For accurate quantitation, individual samples are counted in a gamma-counter.

Antibodies can be labeled with ^{125}I using the techniques described on p. 324. To be useful in most of these assays, they should be labeled to specific activities of 10–100 $\mu Ci/\mu g$. Protein antigens can be labeled to similar activities using the same techniques. As a starting point, add 50,000 cpm/well for microtiter plate assays and 200,000 cpm/cm^2 for sheet assays.

Biotin-Labeled Antibodies, Antigens, or Secondary Reagents

Biotin-labeled reagents normally are coupled to proteins through free amino groups; so as long as the amino groups do not form an essential structural region of the protein, biotin will not interfere with the immunoassay. The major advantage of the biotinylated reagents is that they are stable for years with little or no loss of specific activity. Methods for biotinylating protein antigens are discussed on p. 340.

Biotin-labeled reagents are detected and quantitated by using avidin or streptavidin. These proteins bind tightly to biotin, forming an essentially irreversible complex. Both avidin and streptavidin can be labeled with iodine or enzymes and are available commercially. Usually, streptavidin is the preferred ligand because of its more favorable pI.

Follow the instructions for iodine- or enzyme-labeled reagents on pp. 591 and 592 for detection protocols.

*Enzyme-Labeled Antigens, Antibodies, or Secondary Reagents**

Enzyme-labeled reagents are detected using chromogenic substrates. For assays carried out in microtiter wells, a soluble substrate that is converted to a soluble colored product is used. Enzyme levels are determined by monitoring color development in a spectrophotometer. Many commercial spectrophotometers are now available that will directly read samples in 96-well microtiter plates. For these assays, most workers substitute polystyrene plates for polyvinylchloride, as polystyrene can be read accurately in 96-well spectrophotometers. The polystyrene plates are handled identically to the polyvinylchloride (PVC) plates, except they cannot be cut. Antibodies can be purchased commercially or they can be labeled using the techniques on p. 342. Antigens can be labeled using similar techniques.

For assays carried out on nitrocellulose or diazotized paper, a soluble substrate that yields an insoluble colored product at the site of reaction is used. These results can be evaluated by visual inspection and quantitated by reflection densitometry. Suitable substrates of both types are listed below for the commonly used enzyme labels. For a starting point, use 5–50 ng of antibody-conjugate/well for microtiter plates assays (1/1000–1/10,000 dilutions of most commercial stocks) or 20–200 ng/cm^2 for sheet assays (1/100–1/1,000 dilutions of commercial stocks).

Caution Some chromogenic substrates may be carcinogenic. Consult your local authorities for handling and disposal procedures.

*Engvall and Perlmann (1972); Van Weemen and Schuurs (1971).

HORSERADISH PEROXIDASE—SOLUBLE PRODUCT

The most sensitive chromogenic substrate for detection of horseradish peroxidase (HRP)-labeled reagents is tetramethylbenzidine (TMB).

1. Prior to the detection, dissolve 0.1 mg of 3′,3′,5′,5′-tetramethylbenzidine (TMB) in 0.1 ml of dimethylsulfoxide. Add 9.9 ml of 0.1 M sodium acetate (pH 6.0). Filter through Whatman No. 1 or equivalent. Add hydrogen peroxide to a final concentration of 0.01%. This is enough substrate for two 96-well microtiter plates.

   Hydrogen peroxide is normally supplied as a 30% solution. After opening, it should be stored at 4°C, where it is stable for 1 month.

2. After the final wash in PBS, add 50 μl of the substrate solution to each microtiter well.

3. Incubate for 10–30 min at room temperature. Positives appear pale blue.

4. Add 50 μl of 1 M H_2SO_4, to every well. Positives now appear bright yellow.

5. Read the results at 450 nm.

HORSERADISH PEROXIDASE—INSOLUBLE PRODUCT

Three substrates are suitable for developing dot blots stained with horseradish peroxidase-coupled antibodies. These are chloronaphthol, aminoethylcarbazole, and diaminobenzidine.

Chloronaphthol

4-Chloro-1-naphthol (chloronaphthol) is relatively insensitive but gives an intense blue-black reaction product in an easily controlled reaction. Assays developed with this reagent are photographed readily, but the color fades noticeably on storage. Background binding of the product to the filter is low.

1. Prepare the stock solution of 4-chloro-1-naphthol. Dissolve 0.3 gram of chloronaphthol in 10 ml of absolute ethanol. The chloronaphthol stock is stable at −20°C for at least 1 year.

2. Just prior to developing the assay, add 0.1 ml of chloronaphthol stock to 10 ml of 50 mM Tris (pH 7.6). A white precipitate will form.

3. Remove the precipitate by filter through Whatman No. 1 filter paper (or equivalent).

4. Add 10 μl of 30% H_2O_2. H_20_2 is generally supplied as a 30% solution and should be stored at 4°C, where it will last about 1 month.

5. Place the washed nitrocellulose sheet in a suitable container. Add 10 ml of substrate solution per 15×15-cm^2 membrane. Develop at room temperature with agitation until the spots are suitably dark. A typical incubation would be approximately 30 min.

6. To stop the reaction, remove the H_2O_2 by rinsing with PBS.

Aminoethylcarbazole

3-Amino-9-ethylcarbazole (AEC) yields an aesthetically pleasing red reaction product. It is slightly more sensitive than chloronaphthol but harder to photograph.

1. Prepare the stock solution of AEC. Dissolve 0.4 gram of 3-amino-9-ethylcarbozole in 100 ml of dimethylformamide. The AEC stock is stable at room temperature for at least 1 year.

2. Just prior to developing the assay, add 0.67 ml of AEC stock to 10 ml of 0.1 M sodium acetate (pH 5.2) with stirring. A small precipitate may form.

3. Filter through Whatman No. 1 filter paper (or equivalent).

4. Add 10 μl of 30% H_2O_2. H_2O_2 is generally supplied as a 30% solution and should be stored at 4°C, where it will last about 1 month.

5. Place the washed nitrocellulose sheet in a suitable container. Add 10 ml of substrate solution per 15×15-cm^2 membrane. Develop at room temperature with agitation until the spots are suitably dark. A typical incubation would be approximately 30 min.

6. To stop the reaction, remove the H_2O_2 by rinsing with PBS.

Diaminobenzidine

Diaminobenzidine (DAB, 3,3',4,4'-tetraaminobiphenyl) is an exceptionally sensitive substrate, yielding a brown reaction product. One drawback to DAB is that it reacts so fast that it is very easy to overdevelop giving a high background.

1. Just prior to developing the assay, dissolve 6 mg of 3,3'-diaminobenzidine (use DAB tetrahydrochloride) in 10 ml of 50 mM Tris (pH 7.6). A small precipitate may form.

2. Filter through Whatman No. 1 filter paper (or equivalent).

3. Add 10 μl of 30% H_2O_2. H_2O_2 is generally supplied as a 30% solution and should be stored at 4°C, where it will last about 1 month.

4. Place the washed sheet in a suitable container. Add 10 ml of substrate solution per 15×15-cm^2 membrane. Develop at room temperature with agitation until the spots are suitably dark. A typical incubation would be approximately 1–5 min.

5. To stop the reaction, remove the H_2O_2 by rinsing with PBS.

Diaminobenzidine with Metal Ion Enhancement (DAB/Metal)

The sensitivity DAB can be enhanced by adding cobalt or nickel ions to the substrate solution. In the presence of these ions, the product is slate black in color and more easily photographed than the unenhanced product. One drawback to DAB is that it reacts so fast that it is very easy to overdevelop, giving a high background.

1. Just prior to developing the assay, dissolve 6 mg of 3,3′-diaminobenzidine (use DAB tetrahydrochloride) in 9 ml of 50 mM Tris (pH 7.6). A small precipitate may form. Add 1 ml of 0.3% (wt/vol) $NiCl_2$ or $CoCl_2$.

2. Filter through Whatman No. 1 filter paper (or equivalent).

3. Add 10 μl of 30% H_2O_2. H_2O_2 is generally supplied as a 30% solution and should be stored at 4°C, where it will last about 1 month.

4. Place the washed sheet in a suitable container. Add 10 ml of substrate solution per 15×15-cm^2 membrane. Develop at room temperature with agitation until the spots are suitably dark. A typical incubation would be approximately 1–5 min.

5. To stop the reaction, remove the H_2O_2 by rinsing with PBS.

ALKALINE PHOSPHATASE—SOLUBLE PRODUCT

Probably the best enzyme–substrate combination for sensitive quantitation of 96-well microtiter immunoassays is alkaline phosphatase–nitrophenyl phosphate (PNPP).

1. Prior to developing the enzyme label, dissolve 10 mg of *p*-nitrophenyl phosphate (PNPP) in 10 ml of 10 mM diethanolamine (pH 9.5) containing 0.5 mM $MgCl_2$. This is enough substrate for two 96-well microtiter plates.

2. After the final wash with PBS, wash the plate twice with 10 mM diethanolamine (pH 9.5) containing 0.5 mM $MgCl_2$.

3. Add 50 μl of the substrate solution to each microtiter well.

4. Incubate for 10–30 min at room temperature.

5. Add 50 μl of stop solution, 0.1 M EDTA, to each well. Positives appear bright yellow.

   Read the plate at 405 nm.

ALKALINE PHOSPHATASE—INSOLUBLE PRODUCT

The bromochloroindolyl phosphate–nitro blue tetrazolium (BCIP/NBT) substrate generates an intense black-purple precipitate at the site of enzyme binding. The substrate solution is stable in the absence of enzyme. The reaction proceeds at a steady rate, thus allowing accurate control of the development of the reaction. This allows the relative sensitivity to be controlled by the length of incubation. The BCIP/NBT substrate characteristically produces sharp spots with very little background coloring of the membrane.

Coupled reagents should be prepared with eukaryotic alkaline phosphatase, as this enzyme is readily inactivated with EDTA. The bacterial enzyme is difficult to stop, causing overdevelopment and leading to high background.

1. Prior to developing the assay, prepare the three stock solutions. NBT: Dissolve 0.5 gram of NBT in 10 ml of 70% dimethylformamide. BCIP: Dissolve 0.5 gram of BCIP (disodium salt) in 10 ml of 100% dimethylformamide. Alkaline phosphatase buffer: 100 mM NaCl, 5 mM $MgCl_2$, 100 mM diethanolamine (pH 9.5).

   All stocks are stable at 4°C for at least 1 yr.

2. Just prior to developing the assay, prepare fresh substrate solution. Add 66 μl of NBT stock to 10 ml of alkaline phosphatase buffer. Mix well and add 33 μl of BCIP stock. Use within 1 hr.

3. After the final wash, rinse the sheet twice with 100 mM NaCl, 5 mM $MgCl_2$, 100 mM diethanolamine (pH 9.5). Add 10 ml of substrate solution per 15 × 15-cm^2 membrane. Develop at room temperature with agitation until the spots are suitably dark. A typical incubation would be approximately 30 min.

4. To stop the reaction, rinse with PBS containing 20 mM EDTA, chelating the Mg^{2+} ions.

DESIGNING IMMUNOASSAYS

In addition to the specific assay protocols described above, many different types of immunoassays are possible. The unique specificity of the antibody–antigen interaction and the ease with which each can be labeled permit a wide spectrum of assay designs. The techniques described above are commonly used because they have succeeded in a wide variety of applications. In special circumstances, alternative designs may be more appropriate. The design of these assays will be dictated by the specific circumstances of the individual study. Common techniques that may be useful include different binding supports, different detection methods, and different assay geometries. Suggestions for these are discussed below.

Assay Geometry

A series of summary diagrams illustrates the range of available designs (Fig. 14.4).

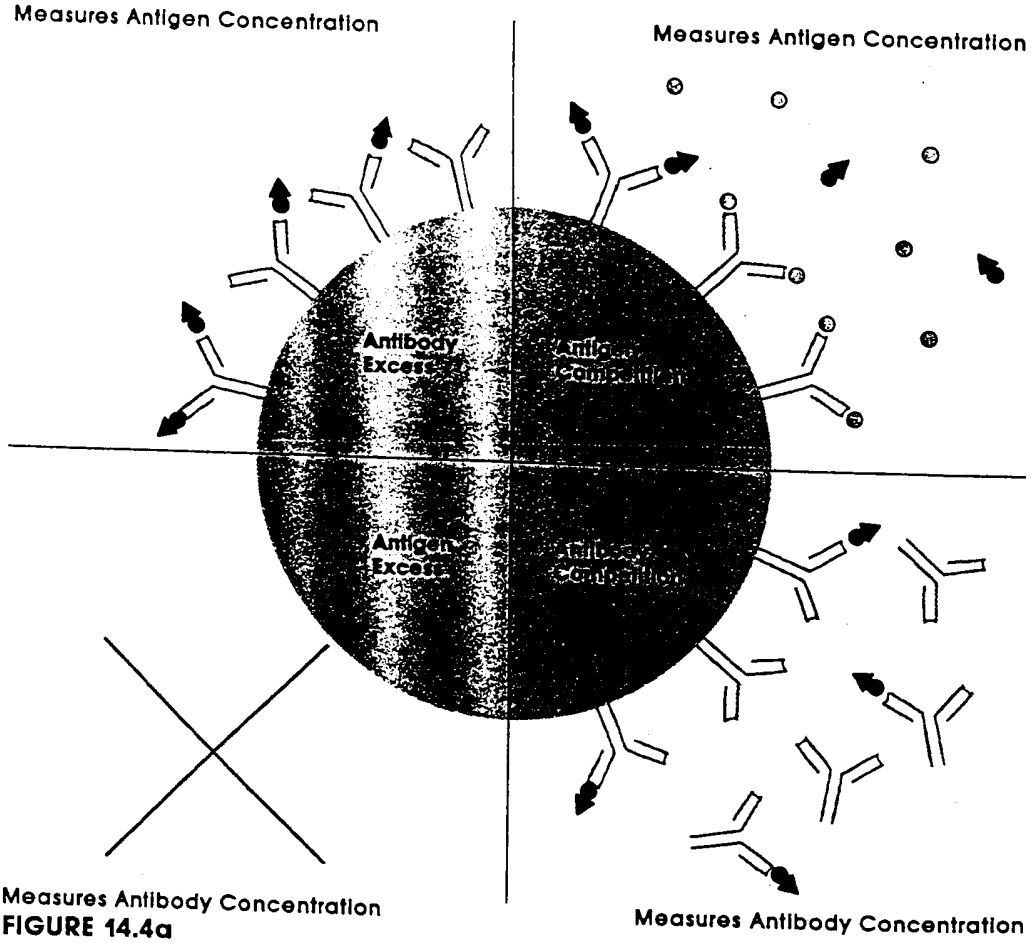


FIGURE 14.4a
Antigen-capture assays, direct detection.

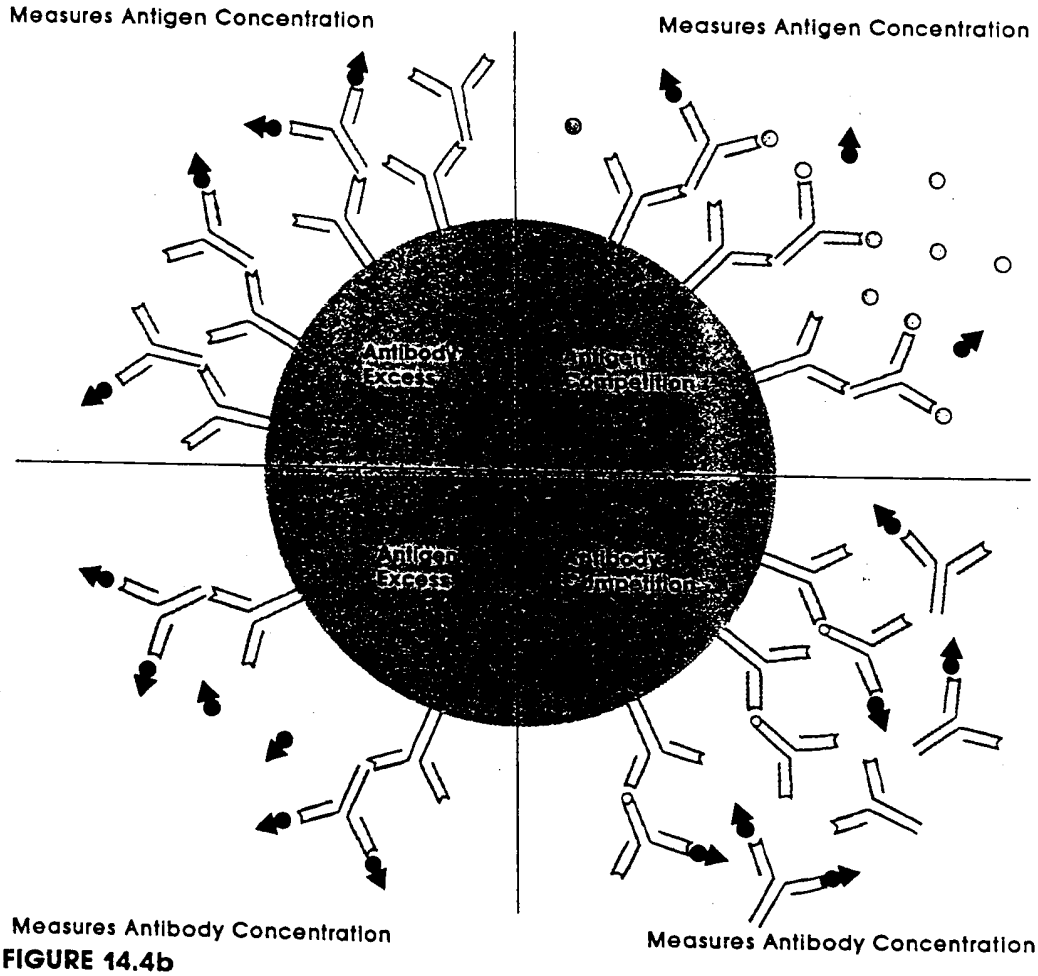


FIGURE 14.4b
Antigen-capture assays, indirect detection.

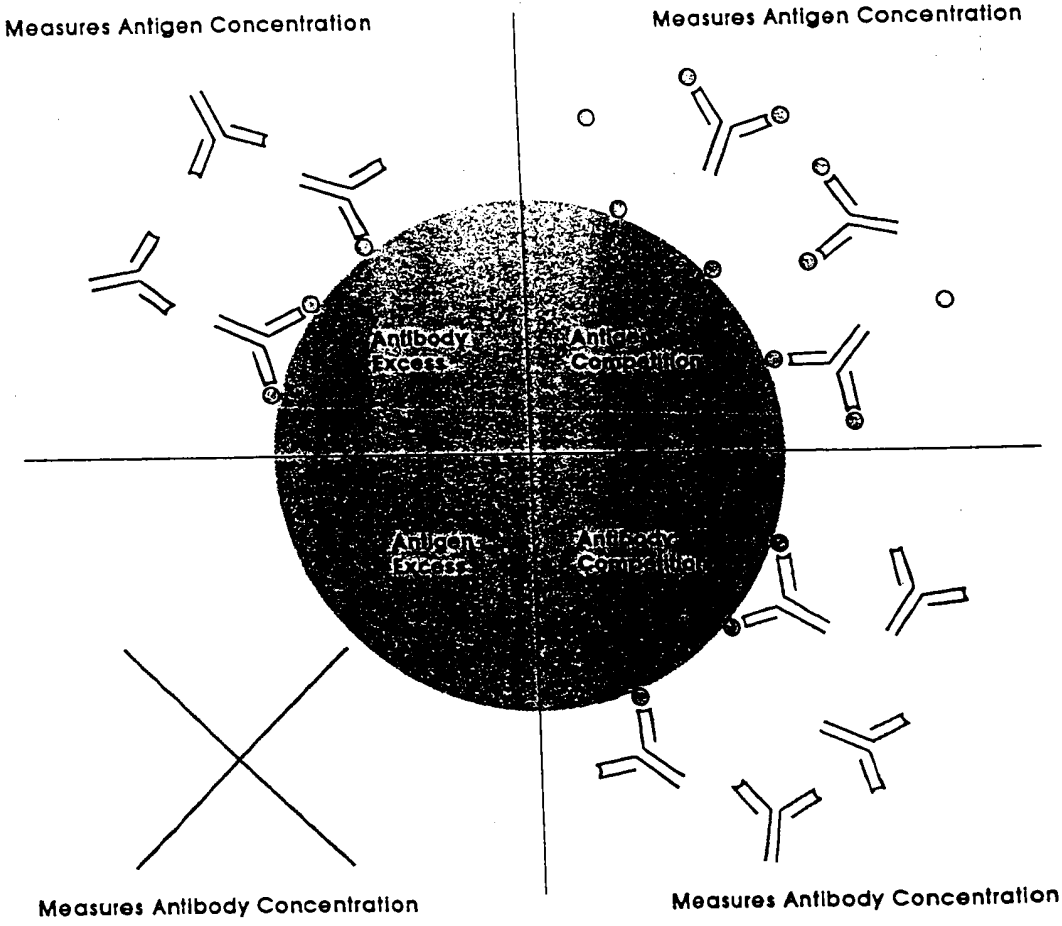


FIGURE 14.4c
Antibody-capture assays, direct detection.

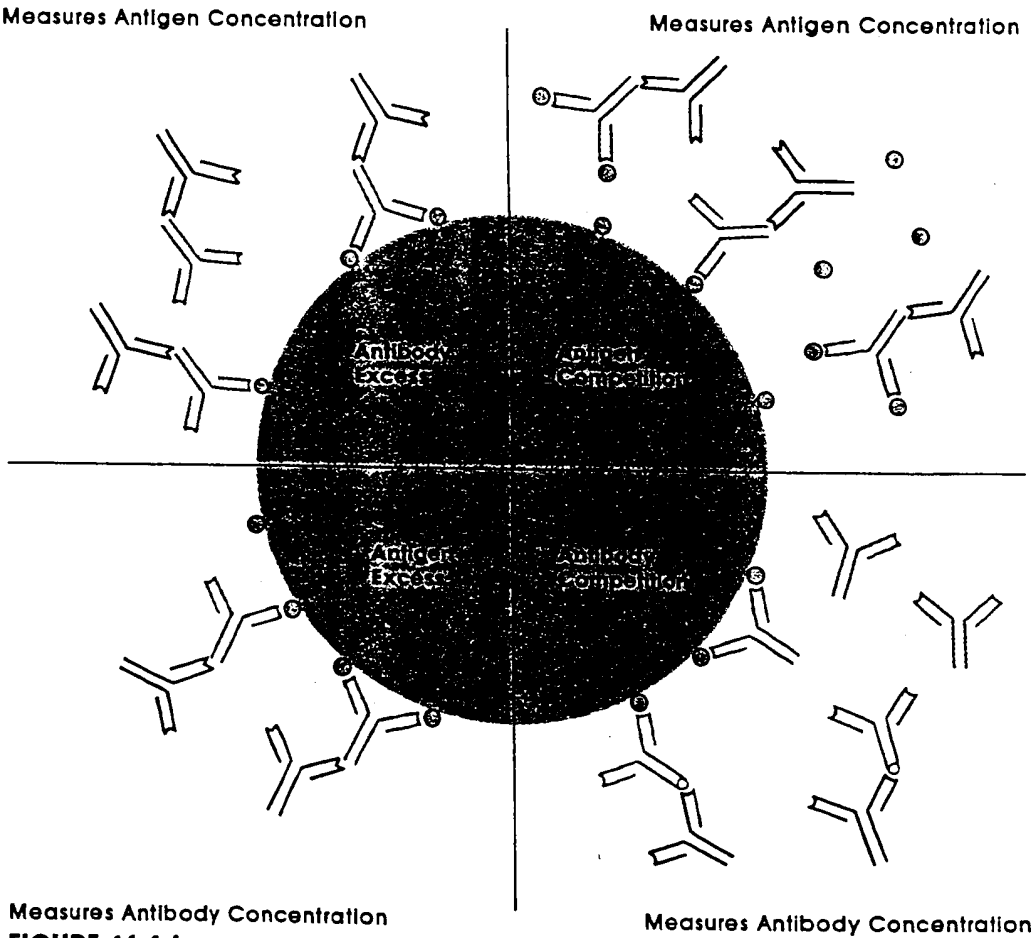


FIGURE 14.4d
Antibody-capture assays, indirect detection.

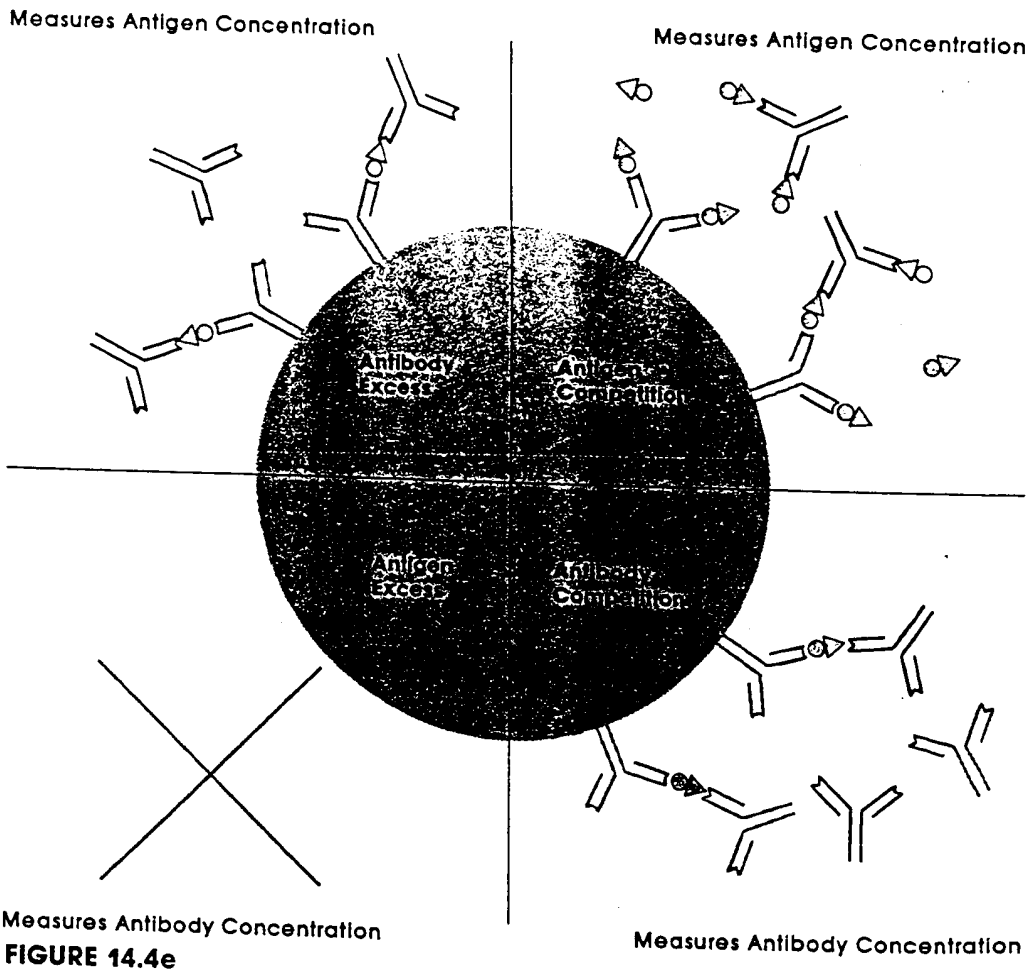


FIGURE 14.4e
Two-antibody assays.

Solid-Phase Matrices for Immunoassays

To allow the easy manipulation of the antibody–antigen complexes during the steps of an immunoassay, one component of the reaction is often bound to a solid substrate. The commonly used solid phases are listed in Table 14.2. Depending on the type of support and on the desired conditions, the solid support can be used in a number of different forms. Commonly, these will be microtiter plates where each well serves as a separate incubation chamber, flat sheets or membranes where the crosstalk between different reactions is limited by diffusion rates, or beads where the individual reactions are performed in separate tubes. Many of the individual matrix materials can be purchased in several of these forms, and the materials for each solid phase are discussed separately below.

TABLE 14.2
Solid Supports for Immunoassays

Support	Forms available	Method of binding	Protein capacity
Nitrocellulose	Membranes Microtiter wells	Noncovalent	100 $\mu g/cm^2$
Polyvinylchloride	Microtiter plates, Sheets	Noncovalent	300 ng/cm^2
Polystyrene	Beads, Microtiter plates	Noncovalent	300 ng/cm^2
Diazotized paper	Sheets	Covalent, through free amino groups	>10 mg/cm^2
Activated beads, numerous attachments possible	Beads	Covalent, through free amino groups	10 mg/ml
S. aureus, Protein A beads	Beads	Noncovalent, although cross-linking possible	20 mg/ml, but antibody linkage only

NITROCELLULOSE*

Both antigens and antibodies can be attached to nitrocellulose by incubating the proteins with the membrane for short periods of time at room temperature. Nitrocellulose binds proteins by noncovalent bonds, but the types of interactions are not clearly understood. However, for many purposes and applications, the bonds are stable, thus making nitrocellulose one of the most useful of the solid phases for immunoassays. The antibodies and antigens are not oriented with respect to their binding sites, and therefore some binding sites will be obscured. Nitrocellulose is available either as sheets or sealed to the bottom of wells in microtiter plates.

1. Cut the nitrocellulose to the appropriate size. Multiple assays can be performed on the same sheet by spacing each assay center approximately 3 to 4 mm apart. This is far enough to avoid cross-contamination between different samples due to diffusion (with 1 μl of sample). Nitrocellulose paper can be used in a dot blot apparatus by cutting it to the dimensions of the apparatus.

2. The concentration of the antibody or antigen solution will be determined by the individual assay conditions. Nitrocellulose will bind approximately 100 $\mu g/cm^2$. The protein can be bound in many different buffers and under many different conditions. Binding buffers should not contain extraneous proteins or detergents such as Tween 20 (low concentrations of SDS, NP-40, or Triton X-100 are fine). If no other factors need to be considered, do the binding in 10 mM sodium phosphate (pH 7) or PBS.

   If the antigen or antibody is plentiful, it can be bound to the entire sheet, regardless of how the paper will be used. Use 0.1 ml/cm^2. Incubate for 2 hr or overnight at room temperature in a humid atmosphere.

   If the antigen or antibody is scarce or expensive, bind it only to the areas where the test solution will be applied. This can be done in two ways, by direct application or by using a dot blot apparatus. When using a dot blot apparatus, place the sheet in the apparatus, and apply the antigen or antibody to just the region of the nitrocellulose that is exposed. Use 30 μl/well. If not using a dot blot apparatus, mark the sheet with a soft lead pencil to show the area where the assay solutions will be added. These spots should be at least 3 mm apart. Add the antigen or antibody at 1 μl/spot. Incubate for 30 min to 1 hr in a humid atmosphere.

*Sharon et al. (1979).

3. Block the remaining sites for protein binding on the nitrocellulose by incubating the sheet in blocking buffer for 2 hr at room temperature or overnight at 4°C.

   There are a number of possible blocking buffers. The two most useful are 3% BSA/PBS with 0.02% sodium azide or Blotto/Tween (5% nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in PBS). The advantages and disadvantages of these and other blocking buffers are listed on p. 496.

NOTES

i. Nitrocellulose is the matrix of choice for dot blot assays. If high protein binding or noncovalent linkage is needed for dot blots, use diazotized paper (p. 609).

ii. Nitrocellulose can be prepared up to 1 week before the assay and stored at 4°C in PBS with 0.02% sodium azide. Sheets may be frozen at this stage and stored at −70°C. Alternatively, many antigens survive indefinitely on dry sheets of nitrocellulose.

*POLYVINYLCHLORIDE OR POLYSTYRENE**

Polyvinylchloride (PVC) or polystyrene will bind antibodies or antigens when the proteins are simply incubated with the plastic. The bonds that hold the proteins are noncovalent, but the exact types of interactions are not known. Because these plastics can be molded into many shapes, they are a convenient solid support for many immunoassays. They are most commonly used in the 96-well microtiter plate form, where the wells provide convenient reaction chambers for immunoassays. Because PVC can be cut easily, it is an excellent matrix for radioimmunoassays in which quantitation is done by counting individual wells. Because it is not translucent, enzyme assays that will be quantitated by a plate reader should be performed in polystyrene and not PVC plates. Polystyrene is also available in many different bead sizes, where it can be used conveniently to bind antibodies or antigens for bead-based assays.

1. Bind the antibody or antigen to the bottom of each well by adding approximately 50 μl of protein solution in each well. Incubate for 2 hr at room temperature in a humid atmosphere. PVC and polystyrene will bind approximately 100 ng/well (300 ng/cm^2).

   The binding can be done at pHs between 6 and 9 at temperatures between 4°C and 37°C. For convenience, the binding normally is done at room temperature at neutral pH. Avoid detergents and extraneous proteins.

2. After the antigen or antibody is bound, the remaining sites on the plate must be saturated by incubating the sheet with any of the blocking buffers described on p. 496. Fill the wells to the top with blocking buffer and incubate at room temperature for at least 2 hr. If no experimental reasons suggest otherwise, use Blotto/Tween (5% nonfat dry milk, 0.2% Tween 20, 0.02% sodium azide in PBS) or 3% BSA/PBS with 0.02% sodium azide.

NOTE

i. Plates may be prepared up to 1 week before the assay. Store in PBS with sodium azide at 4°C. Alternatively, most antibodies and some antigens survive indefinite storage at −20°C after shaking the plate dry.

*Adapted from Catt and Tregear (1967).

*DIAZOTIZED PAPER**

Diazotized paper can be used for dot blot assays in which the amount of protein bound to the sheet must be particularly high. The linkage to the paper is covalent through free amino groups on the antigen or antibody. There are a number of commercially available sources for diazotized paper. The most stable of the derivatized papers is aminophenylthioether cellulose (APT paper). Diazotized paper is prepared by treating the APT paper with an acidic nitrite solution converting it to diazophenylthioether (DPT) paper. The coupling is then done by adding the protein solution to the paper.

1. Cut the APT paper to the appropriate size. If using the paper for multiple assays on the same sheet (dot blots), each assay should be approximately 3–4 mm apart. This is far enough to avoid cross-contamination between the different assays from diffusion of 1 μl of sample. If using the diazotized paper in a dot blot apparatus, cut to the dimensions of the apparatus.

2. Activate the paper by following the manufacturer's instructions or as below. All reactions should be done on ice.

   Wash the APT paper twice with ice-cold distilled water. Drain well and add ice-cold 1.2 M HCl (0.3 ml/cm^2). Add 0.3 ml of 10 mg/ml sodium nitrite (prepared fresh) per 10 ml of HCl. Incubate on ice with occasional rocking for 30 min. Wash twice with ice-cold distilled water. The paper is now ready for binding.

3. Immediately, bind the antigen or antibody to the activated paper by incubating the protein solution with the paper for 1 hr at room temperature. Diazotized paper will bind more than 10 mg/cm^2. The protein must be bound in a buffer that has no free amino groups. Binding buffers should not contain extraneous proteins. If no other factors need to be considered, do the binding in 100 mM sodium phosphate (pH 7).

4. Block the remaining sites for protein binding on the paper by incubating the sheet in 0.2 M ethanolamine (pH 7) for 2 hr at room temperature or overnight at 4°C.

NOTE

i. Diazotized antibody or antigen paper can be prepared up to 1 year before the assay and stored at 4°C in PBS with 0.02% sodium azide.

*Renart et al. (1979) and Reiser and Wardale (1981) based on Alwine et al. (1977).

ACTIVATED BEADS

A number of different types of beads can be activated by chemical treatment to produce an appropriate binding site for proteins with free amino groups. The various types of these activated beads are discussed in detail in Chapter 13 (p. 528). The major advantage when using activated beads is that the antigen or antibody is attached covalently, thus ensuring a stable reactive phase. The disadvantages are caused by the use of the beads which makes washing of numerous samples a time consuming and tedious process. Consult Chapter 13 for the proper binding and blocking conditions.

PROTEIN A BEADS

In some assays protein A beads may be used to bind antibodies to a solid substrate. The protein A has a high affinity for the Fc region of some antibodies and thus can be used to bind antibodies without interfering with the antigen combining site. Protein A and its use are discussed in Chapter 15 (p. 616).

Alternative Detection Methods

Detection with Microscopic Particles

Purified antigens or antibodies can be attached readily to a range of particles such as red blood cells or colored latex beads. Antibodies labeled in this way can be used to detect antigens on dot blots, in microtiter plates, or on immunoblots. While this form of labeling is not as sensitive or quantitative as those described above, it does have a number of advantages. No special apparatus is required, and the results can be scored by visual inspection. Because the label does not need to be developed with a substrate or counted, the protocols are simplified and the results obtained rapidly from very large numbers of samples.

Agglutination

When particles as described above are coated with specific antibody, adding a solution containing multivalent antigens to a suspension of the particles will lead to cross-linking and aggregation (agglutination). These aggregates are detected by simple visual inspection. Similarly, suspensions of antigen-coated particles are agglutinated by solutions containing specific antibody. Agglutination assays can be established using essentially all the assay geometries described above.